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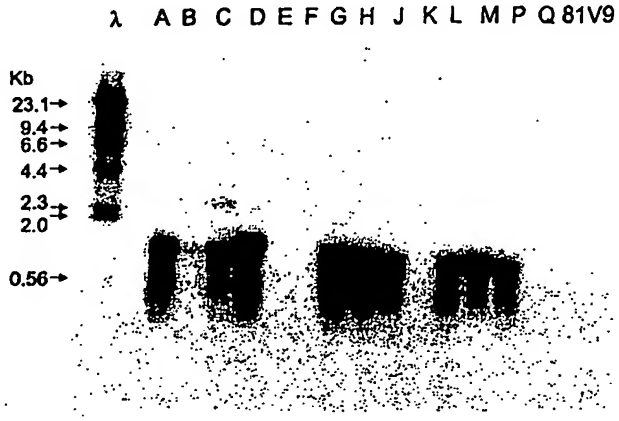

(57) Abstract

A method for preparing a porcine reproductive and respiratory syndrome virus protein is disclosed. This method involves obtaining a gene encoding the desired protein, then inserting the gene within a vector suitable for transforming a plant and transforming a plant with the vector. Following growth of the transformed plant, the desired tissues may be harvested and the protein is extracted from the harvested tissue. This invention is also directed at a method of immunizing pigs against porcine reproductive respiratory syndrome comprising harvesting the transformed plant to obtain harvested tissue and feeding the harvested tissue to pigs, or extracting the protein from the harvested tissue and administering the protein to the pig as a food supplement, or as an injection. However, direct grazing of the plant tissue prior to harvested may also be employed as desired.

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PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME ORAL VACCINE PRODUCTION IN PLANTS

The present invention relates to the production of an animal vaccine in plants. More specifically, this invention relates to the expression of at least one gene obtained or derived, from a porcine reproductive and respiratory syndrome virus. This invention also relates to the purification and administration of corresponding protein(s) to induce the production of protective antibodies in pigs.

BACKGROUND OF THE INVENTION

Porcine reproductive and respiratory syndrome (PRRS) has emerged as an important infectious disease of pigs. Since the disease was first recognized in the United States in 1987 (Keffaber 1989), PRRS has spread rapidly and is now serious problem in the pig industry worldwide. In one area of Iowa, the disease resulted in 85,000 deaths, and in the winter of 1990/91 in Europe, more than one million deaths were estimated to have occurred (Anon 1990, see Meredith 1995 for review). In Canada, PRRS infection is more prevalent in Ontario and Quebec, but outbreaks have been also reported in other provinces (Morin and Robinson 1991, Dea et al. 1992, Sanford 1992). Clinical effects vary from severe outbreaks to persistent infections. Severe symptoms in breeding sows include abortion, premature farrowing, and stillbirths, while in piglets respiratory illness is more common causing preweaning mortality of up to 80% (Loula 1991, see Chritianson and Joo 1994 for review). Persistently infected animals may be clinically asymptomatic, but continue to shed virus and are an important source of virus transmission to naive animals. In severe outbreaks, affected breeding farms can lose an average of 10% of their annual weaner production. Direct losses from PRRS virus infection have been estimated to range from $70 to $350 per sow, and the loss of potential profits is an additional $440 per sow (Meridith 1995, Muirhead 1992, Polson 1990). Losses from grower/finisher pigs due to the reduced growth rate result from an increase in average time (up to 7 days) required to reach slaughter weight. Furthermore, many countries ban importation of

live pigs and semen from affected countries which has created further economic loss among Canadian pig exporters Anon 1992). It is imperative to develop appropriate control measures to prevent PRRS virus infection so that the pig industry in Canada, and particularly Ontario where there are large numbers of pig farms, remains viable and competitive.

Clinically, the majority of pigs become immune to further PRRS virus infections after recovering from primary infections. Sows immunized with inactivated PRRS virus were also protected from abortions. These observations indicate that active immunization is able to induce a neutralizing antibody response important for protection of pigs from PRRS virus infection (Albina 1992). Two types of vaccines are generally considered for active immunization of animals; live-attenuated vaccines and killed vaccines. Live attenuated vaccines are generally more immunogenic, such that higher titer antibody response and long-lasting immune response are elicited. However, conventional live attenuated vaccines are produced by ill-defined mechanisms such as spontaneous mutations or random mutations. Consequently, live vaccines contain a potential of reversion of the vaccine strain to virulence and actual disease production upon vaccination. In contrast, inactivated killed vaccines are safe, but are generally poor in immunogenicity and require a large dose of antigen. Therefore, it is too costly to produce such vaccines specifically for veterinary use. Modern recombinant DNA technology will allow us to develop safe, efficacious, and cost-effective vaccines by producing recombinant subunit PRRS virus antigen in plants. The development of transgenic plants that contained a mammalian gene protein was a major advance in plant biotechnology (Lefebvre et al.1988). Since then numerous mammalian genes have been expressed and plants have been recognized as efficient, low-cost, non-sterile bioreactors that have enormous potential for the production of proteins valuable to both medicine and industry. Plants are higher eukaryotes and are able to properly fold and glycosylate proteins that bacterial systems cannot. Pigs can be fed with the plants expressing PRRS viral antigen and thereby become immune to the viral disease. Our approach will be useful in developing future veterinary vaccine candidates not only

protecting pigs from PRRS virus infections but also for many other infectious diseases in animals

Porcine reproductive and respiratory syndrome (PRRS) is a newly identified infectious disease in pigs and has become the single most important disease in the pig industry. The causal agent for PRRS has been recently isolated, and it appears to belong to a unclassified group of viruses known as the Arterivirus group (Plagemann and Meonnig 1992, Meulenberg et al. 1993). Arteriviruses also include three additional viruses: equine anemia virus, simian hemorrhagic fever virus, and lactate dehydrogenase elevating virus of mice. PRRS virus in pigs is an enveloped virus containing a 15 kilobase genome of single-stranded RNA with positive polarity. The entire genomic sequence has been recently determined using a prototype Lelystad virus isolated in Europe (Meulenberg et al. 1993). The PRRS virus and other arteriviruses appear to share the genomic organization and expression strategy of coronavirus (Snijder et al 1994). Eight open reading frames (ORFs) were identified in the viral genome. It is believed that ORF1a and 1b encode RNA polymerase, which is required for replication and transcription of viral genome. ORF7 encodes a nucleocapsid protein which interacts with and encapsidates viral genomic RNA. Computer predictions have suggested that ORF2 through 6 encode membrane proteins associated with the viral envelope. Among these potential membrane proteins, ORF5 is a major structural protein that has been identified in the purified virions (Loemba et al. 1996). ORF6 is also a membrane protein, but it is unlikely to be an antigen for inducing a neutralizing antibody response as shown in other Arteriviruses. ORF2, 3, and 4 proteins are not characterized, and therefore their biological functions are largely unclear at present. For lactate dehydrogenase elevating virus of mouse, studies using monoclonal antibodies have demonstrated that the ORF5 protein is a major viral antigen inducing neutralizing antibodies (Plagemann and Moennig 1992). It has also been reported that ORF2 may play a minor role in inducing neutralizing antibody in mice.

The production of PRRS virus proteins in an epithelial cell, or tissue culture systems are disclosed in JP 08205858, and US 5,510, 258, respectively. Similarly U.S.

5,510,258 provides a tissue culture base system for the production of PRRSV. In WO 96/04010 and WO 94/18311 a green monkey kidney cell line is used for the production of the virus, and in WO95/31550 the vaccine is produced using a baculovirus expression system. Furthermore, EP732340 discloses the production of a glycosylated form of PRRSV using a suitable host system, with the preferred host as baculovirus. This latter application is also directed to the expression of ORFS2-4. WO96/21012 is directed to the use of alfalfa and tobacco as a plant expression systems for producing immunoglobulins. None of the above publications disclose, or suggest, the preparation of proteins obtained from PRRS virus using a plant host.

A bacterial antigen (*E. coli* enterotoxin) produced in transgenic plants was shown to effectively immunize mice when the crude protein extracts from the transgenic plant tissue were administered orally (Haq et al. 1995). Plants have also been used to produce vaccine antigens for such viral diseases as hepatitis B, and Norwalk virus (Mason et al. 1992, 1996). These antigens were orally immunogenic in mice and comparable to, in the case of the Norwalk virus antigen, to that produced by baculovirus infected insect cell cultures. Plants were transformed using *Agrobacterium* mediated transformation and the antigen was administered by direct feeding of plant tissue or as a crude protein extract.

Plant derived vaccines against such animal diseases as mink enteritis virus and human diseases such as polio have been produced through the expression of viral epitopes on the surfaces of plant viruses, followed by infection with the modified virus of a susceptible host (Dalsgaar et al. 1997, Haynes et al. 1986). The plant virus is purified from the tissue and administered to the test animals. Although this system is very effective, the size of the antigen that can be produced is limited to 37 amino acids, which requires that the epitope mapping of antigen be complete (Yusibov et al. 1997) This is not always the case, especially with newly discovered diseases and expression of full length proteins is the only option. Furthermore, expression is transient and containment is a problem at the agricultural level.

Dramatic evidence of the utility of plants as bioreactors was presented in two recent papers, the first of which demonstrated that the four chains of a secretory immunoglobulin were properly expressed and assembled in plants, and that the antibody was fully functional (Ma et al. 1995). New work has extended the concept human haemoglobin, and further confirmed the utility of plants as bioreactors (Diercyk et al. 1997). These examples have been based on *Agrobacterium* mediated transformation, which allows for the development of stable transgenics that can be reproduced from seed as required.

Although plants have long been sources of pharmacologically active products, the advent of plant genetic engineering has created a myriad of new possibilities (Ma and Hein 1995; Goddjin and Pen 1995). Tobacco has proven itself to be particularly amenable to transformation with heterologous genes and for some time has been the model system for plant transformation (Horsch et al. 1989). Despite the fact that these crop-protection-focused biotechnologies have not found application in tobacco production, a major role does remain for tobacco as a bioreactor. Tobacco leaves are capable of producing high levels (8-10%) of soluble protein (fraction 1 protein, F1P) (Woodleif et al. 1981) and pilot systems have been developed to purify this fraction for use as a high-protein dietary supplement (Montanari et al. 1993). We have adapted the F1P system to transgenic tobacco expressing the sea raven typeII antifreeze protein and defined the agricultural conditions that will maximize transgene protein production (co-pending Canadian application 2,188,220; Kenward 1995). Any potential nicotine contamination in the final product was eliminated by basing the system on a locally adapted tobacco mutant (81V9) that has only a limited capacity to synthesize tobacco alkaloids (Chaplin 1977). This is especially relevant when considering the potential for oral delivery of a transgene protein as edible plant tissue.

Alfalfa presents some unique advantages as a bioreactor for protein production in plants. Protein levels are typically in the 20% range on a whole-plant basis and can be higher depending on management. This feature, coupled with high vegetative yields, enables a crop of alfalfa to produce more protein per hectare than any other crop in

Ontario. This is largely due to the very efficient fixation of nitrogen by Rhizobia in root nodules, for which the leaves act as a strong sink. As a result, high protein levels are reached in planta without the cost and environmental impact of nitrogen fertilizers. As alfalfa is also a perennial, a stand of alfalfa provides additional environmental benefits such as retention of snow (and moisture) in the field and reduction of wind and soil erosion; the perennial habit also eliminates the costs of tillage and planting required for annual crops.

As the production of plant recombinant proteins moves from the bench to pilot scale regulatory issues must be addressed (Miele 1997). From both a regulatory and public safety standpoint non-food species are ideal for the production of biologically active proteins. Tobacco and alfalfa are non-food crops and therefore the risk of accidental leakage of transgenic plant material expressing genes for biologically active proteins into the human food chain is near zero. Other plant bioreactor systems do not offer this advantage. Producing the tobacco in Canada, where there are no naturally occurring wild *Nicotiana* species, further minimizes the risk of gene leakage to the local flora. With the tobacco system, protein production is based on leaves, not seed or tubers, and when coupled with the fact that the leaves are harvested before flowering there is virtually no risk of uncontrolled bioreactor plants occurring in future crop seasons. Tobacco does not over-winter in Canada and there is therefore no rattooning the following season. The background genotype and the system used for transgene protein production addresses a number of potential regulatory concerns and begins to deal with the issue of biologically active secondary metabolites that may impair product quality.

The present invention provides a means of low-cost production of a viral antigen protein capable of inducing production of protective antibody against PRRS virus in pigs.

SUMMARY OF THE INVENTION

The present invention relates to the expression of an animal vaccine in plant tissue. More specifically, this invention relates to the expression of at least one gene obtained from a PRRS virus in plants and the administration of this gene product to pigs.

According to the present invention there is provided a method of preparing at least one porcine reproductive and respiratory syndrome virus protein comprising the step of obtaining a gene encoding the protein, inserting the gene within a vector suitable for transforming a plant, transforming a plant with the vector, and growing the transformed plant. This method may use a native gene sequence, or a synthetic gene sequence. Furthermore, this invention is directed to the above method wherein the transformed plant is harvested to obtain harvested tissue, extracting the protein from the harvested tissue, and optionally purifying the protein following the step of extraction. This invention also provides for a protein that is prepared by this method.

This invention is also directed to the above method, wherein the native, or synthetic gene sequence is modified in order to enhance expression of the gene and stability of the encoded protein, within plant tissues. This invention also includes the above method, wherein the modification includes replacing the native signal sequence with a heterologous signal sequence, an ER retention motif, a cleavage site, and a HIS tag.

This invention also provides for a method as described above, wherein the gene encodes the ORF5 protein.

This invention is also directed to the method described above wherein the plant is a tobacco plant or an alfalfa plant.

Also considered within the present invention, is a DNA molecule defined by SEQ ID NO:1, and a vector comprising this DNA molecule operatively associated with a promoter, enhancer and terminator regions. Furthermore, this invention provides for a plant comprising the above defined vector.

This invention is also directed to a method of immunizing pigs against porcine reproductive respiratory syndrome comprising transforming a plant with the vector defined above, growing the transformed plant, harvesting the transformed plant to obtain harvested tissue, feeding the harvested tissue to pigs, and repeating step of feeding , as needed. Also included within the provisions of this invention is the method as defined wherein a protein encoded by the DNA molecule present within the vector is extracted from the harvested tissue and administered to the pig as a food supplement, or as an injection. Furthermore, this invention is directed to administering non-harvested plant tissues to a pig.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 presents an example of the nucleotide sequence of a synthetic PRRS virus ORF5 gene. Where changes were made to the native mature protein sequence the original base is presented above. The PR1b secretory signal from tobacco is found from bases 1 through 93, the mature PRRS virus ORF5 coding region, from bases 94 through 621, a thrombin cleavage site from bases 622 through 636, a histidine tag from bases 649 through 666 and a KDEL motif at bases 667 through 678.

FIGURE 2 represents an example of a simplified cloning scheme used to construct a synthetic PRRS virus ORF5 gene from oligonucleotide blocks. Blocks I to III represent individual oligonucleotides that are first annealed then ligated, then amplified to form DNA duplexes having unique restriction sites that allow for enzymatic assembly of the complete synthetic gene. A Bam H1 restriction site was added to the 5' end of the sequence, and a Kpn 1 sequence added to the extreme 3' end for ease of cloning. The leader sequence from alfalfa mosaic virus is found from bases 10 through 45, the PR1b secretory signal from tobacco is found from bases 49 through 143, the mature PRRS virus native ORF5 protein coding region from bases 144 through 663. The individual base changes made as a part of the codon optimization process or to add restriction sites are exemplified as the single boldface letters above those in the native sequence. The thrombin cleavage site is found from bases 664 through 681, the histidine tag from bases 692 through 709 and the ER retention signal from bases 710 through 721.

FIGURE 3 represents an example of oligonucleotide sequences used to construct one of the blocks that make up the synthetic gene.

FIGURE 4 shows complete sequences of primers comprising Block I, II and III, used for constructing synthetic ORF5. Primers 1 to 12 are SEQ ID NO's:2 to 13, respectively.

FIGURE 5 shows Northern analysis of tobacco plants transformed with a native ORF5 gene construct (pCAMTerX-ORF5A), or a synthetic ORF5 gene construct, (pCAMTerX-ORF5B). Figures 5A and B; Northern blots of RNA obtained from leaf tissue of plants transformed with pCAMTerX-ORF5A. Figures 5 C and D; Northern blots of RNA obtained from leaf tissue of plants transformed with pCAMTerX-ORF5B. "λ" represents the molecular size marker; "81V9" the untransformed (control) plant.

FIGURE 6 shows Western analysis of tobacco plants transformed with a synthetic gene construct (pPSP-ORF5B). Proteins were isolated from plants using standard techniques, separated using SDS-PAGE and transferred onto membrane for probing with a polyclonal antiserum specific for ORF5. The antiserum was obtained from pigs. Lane M is prestained molecular weight markers; Lane 1 an untransformed plant (control); and lanes 2 to 9 transgenic plants.

DESCRIPTION OF PREFERRED EMBODIMENT

This invention is directed to the expression of an animal vaccine in plant tissue. More specifically, this invention relates to the expression of at least one gene obtained from a PRRS virus in plants and the administration of this gene product to pigs.

The present invention provides a means of low-cost production of a viral antigen protein capable of inducing production of protective antibody against PRRS virus in pigs. In order to express the desired protein in plants, a gene encoding the gene of interest of a PRRS virus, for example the ORF5 protein, may be used. It is also contemplated that a synthetic gene encoding the protein of interest, for example the ORF5 protein, is constructed so that its expression product is the same as the native viral protein. In order to optimize the expression of the foreign gene within plants, the native or synthetic gene may be altered or designed, as the case may be, in a way so that the corresponding protein is produced at a level higher than the native gene.

Preferably a synthetic gene will have from about 60 - 90% homology with the native gene and to be designed, that is modified, for high level expression in plants. More preferably, the synthetic gene comprises from about 80 - 90% homology with the native gene. In the examples below, a gene comprising 82.% homology is disclosed, however, it is to be understood that this embodiment is not to be considered limiting in any manner.

It is also contemplated that further modifications of a native or synthetic gene may occur so that expression of the gene, and stability or purification of the protein can be optimized. For example, modification of the 5' or 3' region of these genes can be carried out in order to enhance expression of the gene and target the product to an appropriate intercellular compartment to ensure stability. Below is exemplified modification of a native gene so that both the native and the synthetic gene comprise a KDEL ER retention motif, a PR1b signal sequence, and a cleavage site and histidine tag to aid in purification of the encoded protein. However, these alterations are to be

considered as examples only, as it is contemplated that other 5', 3', or internal modifications may be utilized in order to optimize expression, stability and, optionally, purification of the expressed protein. It is also contemplated that fragments or portions of genes of a desired ORF of the PRRS virus, that encode fragments with antigenic properties, may be expressed within plant tissues.

In order to demonstrate the present invention, native and synthetic genes coding for an ORF5 protein from PRRS virus is exemplified. This synthetic gene has, for example, the nucleotide sequence presented in SEQ ID NO:1 (also see Figure 1), however, other genes encoding ORF's of the PRRS virus, or combinations of these ORF's may easily be substituted for the exemplified synthetic gene (e.g. see Figure 1). It is preferred that the synthetic gene encoding the mature protein comprises a codon bias similar to highly expressed genes found in plants, that the protein carry a motif that allows for simple extraction, and that the immature protein is targeted to a compartment of the cell to enhance stability of the product, for example the lumen of the endoplasmic reticulum (ER). However, other sites are also contemplated for example, extracellular secretion, in order to simplify extraction protocols, or chloroplast or mitochondrial compartments. In the case of targeting to the ER, further *in vivo* processing of the expressed product will take place including cleavage of the signal peptide. The modified native or synthetic genes also comprise a C terminal motif that causes the encoded protein to be retained within the ER.

We have obtained a PRRS virus (PA-8) isolate from an outbreak in a Canadian pig farm and cloned and sequenced 4 kb of the 3' terminal of the viral genome (Yoo 1997). This region contains the complete coding sequence for ORFs 2 through 7. ORF5 consists of 600 nucleotides which encodes a polypeptide of 200 amino acids. The predicted ORF5 protein contains a hydrophobic signal sequence at the N-terminus and two additional hydrophobic domains which are likely the membrane spanning region. Four putative N-linked glycosylation sites were also found in the region of first 50 amino acids. The complete ORF5 sequence was engineered for insertion into the vaccinia virus genome, and we were able to construct a recombinant vaccinia virus (Yoo 1997). This

recombinant vaccinia virus carries the ORF5 gene in the thymidine kinase locus of the viral genome under the control of vaccinia virus late promoter. Upon infection of cells with the recombinant vaccinia virus, a polypeptide of 25 kD in molecular weight has been specifically identified. This polypeptide reacts specifically with monospecific antibody raised against the ORF5 protein produced in E. coli. These results indicate that the ORF5 gene is functional and can be used for protein expression in plant systems. However, it is to be understood that the present invention is also directed to the use of other putative ORF's from the PRRS virus may be used either singly, or in combination with other ORF's as needed.

By "gene", it is meant a particular sequence of nucleotides including the coding region, or fragment thereof, and optionally the promoter and terminator regions which regulates expression of the gene, as well as other sites required for gene expression for example a polyadenylation signal which regulates the termination of transcription.

By "coding region" or "structural gene", it is meant any region of DNA that determines the primary structure of a polypeptide following genetic transcription and translation. Furthermore, fragments comprising regions of interest of a coding region or structural gene may also be employed as needed.

By "synthetic gene" it is meant a DNA sequence of a structural gene that is synthesized chemically. A synthetic gene can comprise a fragment or the entire coding region of the gene of interest. Furthermore, a synthetic gene may also comprise regulatory elements that enhance expression of the gene, or motifs that aid in the stability of the protein product. It is also contemplated that a synthetic gene optionally includes regions useful for the isolation and purification of the protein, or the protein fragment, encoded by the synthetic gene.

By "DNA regulatory region" it is meant any region within a genomic sequence that has the property of controlling the expression of a DNA sequence that is operably linked with the regulatory region. Such regulatory regions may include promoter or

enhancer regions, and other regulatory elements recognized by one of skill in the art. By "promoter" it is meant the nucleotide sequences at the 5' end of a coding region, or fragment thereof that contain all the signals essential for the initiation of transcription and for the regulation of the rate of transcription. The promoters used to exemplify the present invention are constitutive promoters that are known to those of skill in the art. However, if tissue specific expression of the gene is desired, for example seed, or leaf specific expression, then promoters specific to these tissues may also be employed. Furthermore, as would be known to those of skill in the art, inducible promoters may also be used in order to regulate the expression of the gene following the induction of expression by providing the appropriate stimulus for inducing expression. The use of inducible promoters may be required as the use of constitutive synthesis of a foreign protein may in some cases be toxic or inhibit normal growth of the plant, with the result that the only plants which regenerate following transformation are those expressing very low levels of the foreign protein. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor, that binds specifically to an inducible promoter to activate transcription, is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods.

By "constitutive promoter" it is meant a promoter that directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive promoters include those associated with the CaMV 35S transcript and *Agrobacterium* Ti plasmid nopaline synthase gene.

The chimeric gene constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumour inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene of the present construct can therefore be used to construct chimeric genes for expression in plants.

The gene constructs of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide for resistance to an antibiotic such

as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β-glucuronidase), or luminescence, such as luciferase are useful.

By "transformation" it is meant the stable interspecific transfer of genetic information that is manifested phenotypically. The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc as would be known to those of skill in the art.

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

By "codon optimization" it is meant the selection of appropriate DNA nucleotides for the synthesis of oligonucleotide building blocks, and their subsequent enzymatic assembly, of a structural gene or fragment thereof in order to approach codon usage within plants.

The literature describing the process of synthetic gene construction using codon optimization is limited to bacterial genes such as those producing the insecticidal crystal proteins of *Bacillus thuringeinsus* and two insect genes (see Kozeil et al. 1996 for review). The process has been successful for the bacterial genes (Adang et al. 1996,

Sardana et al. 1996 for e.g.). The codon optimized insect gene did not respond to the changes and it was later shown that the need for correct cellular targeting overshadowed any improvements due to codon optimization (Hightower et al. 1994, Florack et.al. 1995). It has also been shown that ER retention signals can dramatically increase the concentration of disulfide bonded transgene proteins, therefore the KDEL ER retention motif was added to the 3' end of the ORF5 cDNA using site directed mutagenesis (Schouten et al. 1996, Kunkel 1985). Although some native signals from mouse that target a protein to the lumen of the ER have been found to function properly in plants this is by no means assured in every case, use of the PR1b signal sequence will however assure correct targeting of a foreign protein and in some cases the use of a plant signal will increase transgene protein concentration (Schouten et al. 1996, Florack et al. 1995, Denecke et al. 1990). No reports describe the expression of foreign genes in plants that comprise the combination of elements as disclosed herein, including the use native plant ER targeting signals, codon optimization and ER retention motifs together in the same synthetic gene.

By ORF5 protein" it is meant one of the known two membrane-spanning envelope glycoproteins found within members of the Arterivirus family.

In order to maximize expression levels and transgene protein production, the ORF5 gene was examined at the DNA level and then the coding region optimized for expression in plants using a procedure similar to that outlined by Sardana et al. 1996. The standard deviation of codon usage, a measure of codon usage bias, was calculated by first finding the squared proportional deviation of usage of each codon of the native ORF5 gene relative to that of highly expressed plant genes, followed by a calculation of the average squared deviation. The formula used is:

$$SDCU = \sum_{n=1}^{N} [(X_n - Y_n)/Y_n]^2/N$$

Where X_n refers to the frequency of usage of codon n in highly expressed plant genes, where Y_n to the frequency of usage of codon n in the gene of interest and N refers to the total number of codons in the gene of interest. A table of codon usage from highly expressed genes of dicotyledonous plants was compiled using the data of Murray et al. (1989).

Assembly of the synthetic PRRS virus ORF5 gene of this invention is performed using standard technology know in the art. The ORF5 preprotein designed for increased expression in plants is assembled enzymatically, within a DNA vector, from chemically synthesized oligonucleotide duplex segments. The synthetic PRRS virus ORF5 gene is then transformed to plant genomes using methods known in the art. It is contemplated that a transgenic plant comprising the heterologous protein may be administered to an animal in a variety of ways depending upon the need and the situation. For example, if the protein is orally administered, the plant tissue may be harvested and directly feed to the animal, or the harvested tissue may be dried prior to feeding, or the animal may be permitted to graze on the plant with no prior harvest taking place. It is also considered within the scope of this invention for the harvested plant tissues to be provided as a food supplement within animal feed. Furthermore, the protein obtained from the transgenic plant may be extracted prior to its use as a food supplement, in either a crude, partially purified, or purified form. The administration of any of these protein forms to pigs, results in the formation of an antibody that protects the animal from the PRRS virus.

EXAMPLES

The following examples while illustrating the embodiments of the invention are not to be considered as limiting the scope of this invention in any manner

EXAMPLE 1

Design of the synthetic PRRS virus ORF5 gene

A full length cDNA corresponding to ORF5 of the PRRS virus strain PA-8 (Yoo 1997) was cloned as a 0.605 bp BamHI fragment into the cloning vector pGEM3zf(+). From that vector the complete nucleotide sequence of the PRRS strain PA-8 native ORF5 gene was determined using standard methods.

The standard deviation of codon usage (SDCU) was calculated and found to be 87.0. Upon replacement of rare codons with those more commonly used in plants, as exemplified in Figure 1 (SEQ ID NO:1), the SDCU was reduced four fold to 20.6.

The synthetic ORF5 gene was constructed by assembling three blocks of DNA of approximately 200 bp each (Block I, Block II, and Block III: see Figures 1 and 2 and sequences listed in Figure 4; Primers 1 to 12 of Figure 4 are SEQ ID NO's 2 to 13, respectively). Each block contained restriction site overhangs at the 5' and 3' ends for cloning purposes using standard techniques and allowing ligation of the synthetic blocks to form the complete ORF5 coding region. The completed ORF5 coding region contained 5' NcoI and BamHI restriction sites, and 3' BspHI and KpnI restriction sites.

The individual blocks of the ORF5 synthetic gene were constructed by designing overlapping oligonucleotides of approximately 60-90 bp in length utilizing codons typically used in highly expressed genes in plants (Fig. 3). The central pair of oligonucleotides (100 pmol each) were denatured together for 1 minute at 94°C, cooled to 60°C over 35 minutes to allow annealing, held at 60°C for 5 minutes at which time 5.25 units of Expand DNA polymerase (Boehringer Mannheim) were added. The reaction mixture was then warmed to 68°C over five minutes and the fill- in reaction proceeded for two hours at 68°C. Ten µl of the resulting double stranded DNA fragment was used as a template for PCR amplification using 10 pmole of the 5' and 3' primers and 5.25 units of Expand DNA polymerase, 350 µmole dNTP's, 1.75 mM $MgCl_2$. The amplified

DNA fragment was then gel purified using standard methods. The PCR fragments corresponding to blocks 1, 2 and 3 were introduced into the pGEMTEasy cloning vector according to the manufactures recommended method. Blocks I, II and III were removed from the pGEMTEasy vector by digestion with restriction enzymes corresponding to the introduced enzyme sites at the 5' and 3' ends of the fragments. The fragments were assembled together into the complete synthetic gene by successive cloning steps into a pBluescript (KS+) cloning vector. For example, Blocks I, II, and III were liberated from the vector by digesting with BamHI and HindIII, HindIII and XbaI, XbaI and KpnI, respectively. Blocks II and III were introduced simultaneously into pBluescript digested with HindIII and KpnI (tripartite ligation). This vector (pBS23) was then digested with BamHI and HindIII and Block I was added to complete the synthetic gene.

Since the ability of the ORF5 signal sequences to correctly target the protein to the lumen of the endoplasmic reticulum (ER) is unknown, the first 29 amino acids in the ORF5 gene were replaced with a putative signal sequence obtained from the tobacco pathogenesis related protein 1b (PR1b) signal sequence. The replacement was effected by designing two alternate primers for the construction of Block I of the codon optimized ORF5 gene. An example of those two primers is presented in Figure 3 (SEQ ID NO:2 and 3; also see primers 1-4 in Block I of Figure 4). Two synthetic genes, one with the PR1b signal and the other with the native signal were compared and the PR1b signal was found to result in higher levels of protein synthesis (data not presented).

In order to facilitate the purification of the ORF5 protein from plant tissue a C terminal thrombin cleavage site and a histidine tag (NOVAGEN, Cambridge) was placed between the end of the native protein sequence and beginning KDEL motif. This sequence was added by modifying the synthetic oligonucleotides used in the construction of Block III. Therefore, gene constructs comprising: a PR1b sequence, Block I-III (synthetic ORF5), cleavage site HIS tag, and a KDEL motif were prepared.

These synthetic PRRS virus ORF5 genes were sequenced and found to encode the same protein as native ORF5 gene, except for the addition of the PR1b sequence, HIS tag sequence, or KDEL motif, as the case may be.

The duplicated 35S enhancer-promoter plus AMV leader sequence (Kay et al. 1987; Jobling and Gehrke 1987) and the p1275 constitutive promoter system from tobacco (B.L. Miki WO 97/282268) were used for construction of vectors comprising the synthetic ORF 5 gene. For the duplicated 35S and the p1276 expression systems the native and codon optimized ORF5 genes were inserted as Nco1-BamH1 fragments, immediately downstream from the promoters and between the T-DNA border sequences. The duplicated 35S vector was named either pCAMTerX-ORF5A or pCAMTerX-ORF5B, depending upon its component elements:

pCAMTerX-ORF5A comprises a native ORF5 gene;

pCAMTerX-ORF5B contains the synthetic ORF5 mature protein coding region, the PR1b signal sequence and the KDEL ER retention motif;

As the 35S promoter is known to be under expressed in alfalfa (Maggio et al. 1996), a parallel set of constructs were also prepared for the expression synthetic ORF 5 genes using the "Purdue Superpromoter" (Ni. et al, 1995, Plant J. vol 7; pp. 661-676). This superpromoter uses subdomains of the *Agrobacterium* octopine and mannopine synthase promoters and activators to drive high level constitutive expression of heterologous genes in plants. The native ORF5, or synthetic ORF5 genes were cloned into the pBI101.2 derived binary vector pBISN1, which carries the superpromoter, to produce pPSP-ORF5A and pPSP-ORF5B:

pPSP-ORF5A comprises the native gene,

pPSP-ORF5B contains the synthetic ORF5A mature protein coding region, the PR1b signal sequence and the KDEL ER retention motif.

These plasmids were transformed into *Agrobacterium* strain EHA105 carrying the disarmed Ti plasmid pEHA104. The plant selectable marker within this plasmid is neomycin phosphotransferase (*nptII*, Van den Elzen et al. 1985). Only the best expressing constructs were used with the p1275 promoter resulting in the vector p1276ORF5. To prepare vector p1276ORF5, the synthetic ORF5 gene cut with 5' NcoI and 3' BspMI was ligated into the pRND400 binary vector containing the p1275 promoter upstream of the NcoI restriction site. The BspHI overhang at the 3' end of the gene and the NcoI restriction site are compatible and ligate together destroying both the sites.

Introduction of the ORF5 genes to plant genomes

The various synthetic ORF5 gene constructs were then transformed into tobacco using *Agrobacterium* mediated transformation using standard protocols within the art (Horsch et al. 1989).

Low nicotine tobacco plants from the cultivar 81-V9 grown aseptically *in vitro* were used as starting material. The use of aseptic *in vitro* material reduces losses due to contamination compared with leaves from greenhouse-grown plants. For transformation, the leaf material with the midvein excised was cut into 1 cm^2 fragments using a scalpel and precultured with the epidermal side down for 2 days on solid media (Media 1) consisting of MS salts, B5 vitamins, 3% sucrose, 1 mg/L BAA (6-benzylaminopurine) and 0.1 mg/L NAA (alpha-naphthalene acetic acid).

Infection with *Agrobacterium* strains each containing a vector comprising one of the five constructs identified above was achieved using overnight cultures grown in LB medium diluted by about 50%. The leaf explants were submerged quickly (about 30 seconds) and blotted on Whatman No.2 filter paper to remove excess bacteria. They are then returned to Media 1 for 2 days. To inhibit bacterial growth and initiate the selection of transformed tissues, the explants are transferred to media with 500

ug/ml Timentin (SmithKline Beecham, Oakville) and the selective agent, 100 ug/ml kanamycin (Sigma, St. Louis). The explants are transferred to fresh media every 2-3 weeks and shoots began to emerge within 3-5 weeks.

Once well-defined stems are developed, the shoots are excised and transferred to Magenta boxes with solid media consisting of MS salts, B5 vitamins, 3% sucrose, 500 ug/ml cefotaxime or Timentin and 100 ug/ml kanamycin. Following root development the putative transgenic plants are transferred to the greenhouse. To ensure that each plant arises from an independent transformation event the explants are divided into separate fragments early in the procedure and only 1 shoot is selected from each fragment.

For alfalfa transformation, an overnight liquid culture of the *Agrobacterium tumefaciens* strain C58C1 carrying the helper plasmid pMP90 and one of the five binary vectors pBISN1ORF5A through E were prepared. The bacteria were subcultured into fresh liquid medium and the culture used when the optical density = 1.0.

Young petioles (3 youngest petioles per stem) were harvested from greenhouse-grown alfalfa plants, and sterilized by soaking in 75% ethanol for 30 seconds followed by soaking in 4% calcium hypochlorite for 20 minutes. The petioles were then rinsed with three changes of sterile water and cut into sections of 1cm in length. The petiole sections were dipped in *Agrobacterium* culture for 1 minute, removed from the bacterial culture and placed directly on induction medium (containing 100 μM acetosyringinone, no antibiotics) and incubated in the dark at 25°C for 2 days.

The petioles were then rinsed in sterile ½ strength MS salt solution (Sigma, pH 5.8) for 2 minutes and transferred to induction medium containing 500 mg/L claforan and 50 mg/L kanamycin. The petioles were transferred to fresh plates approximately every 10-14 days. After several weeks small calli appear which are left until somatic embryos develop. The embryos were germinated on ½ MS media (pH 5.8) containing

50 mg/L kanamycin and 1% (w/v) sucrose. The green, elongated embryos were transferred to germination medium individually, without any attached callus, then laid on the media until roots, shoots and leaves develop. Individual plantlets were then placed in soil in pots and transferred to the greenhouse for further growth.

Each of the putative transgenic tobacco and alfalfa plants were screened for the presence of the transgene using PCR using primers specific to the 5' and 3' ends of the transgene (see Figure 4). For example, the transgene of Fig. 1 would use primers of sequence:

upper primer 5' GCAAATGCCTCAAACGATTCAAGC 3', (Tm 60°C); and
lower primer 3' TCTCAAAGTCGCCTTGTTACCCC 5', (Tm 59°C),

however, other primer combinations may also used (see Figure 4 for other primers). DNA was extracted from plant tissue using standard methods and subjected to 30 cycles of amplification using 15pMol of both primers, 100 mM dNTPs, 2mM MgCl2, 1U Taq polymerase and 50 ng of DNA template. DNA from positive plants was digested with a restriction enzyme that does not cut inside the T-DNA borders and subject to Southern analysis using the requisite transgene as a probe. Probes were randomly labelled using 50 uCi of [α^{32}P]CTP using standard methods. All washes were conducted at high stringency.

RNA was isolated from transgenic plants containing the native ORF5 gene and was analyzed using reverse transcription PCR (RT-PCR), or Northern analysis (see Table 1).

For RT-PCR 10 μg of RNA was first treated with RNase-free DNase and 2 μg was used for cDNA synthesis. Oligo dT (12-18) 05.μg was mixed with the RNA and heated to 90°C, allowed to cool to 70°C, and then chilled on ice. First strand synthesis proceeded for 50 min at 42°C in a buffer containing 10 mM DTT. 0.5mM dNTP, 50 mM Tris HCL pH 8.3, 75 mM KCl, 3 mM MgCl and 200 units of

Superscript II reverse transcriptase (Gibco BRL Life Technolgies). The enzyme was heat-inactivated at 70°C for 15 min. PCR was then done as described above with 2 μL of the reverse transcription reaction as the template and primers specific to ORF5. Transcript for ORF5 was detected in 27 of the 30 plants tested.

Northern blot analysis was done with 20 μg RNA extracted from transgenic plants containing either the native or the synthetic ORF5 gene using standard techniques. The transcripts detected on the Northern blots (see Figure 5 A-D) ranged in size from 1200 to 1400 bases for the native ORF5 and 1500 to 1800 bases for the synthetic ORF5 as compared to λ HindIII fragments run on the same blots. These size estimates are larger than the native gene (602 bp) or synthetic gene (752 bp) presumably because of the additional sequence from the promoter (e.g. 35SCaMV, 250 bp), terminator (nos terminator 250 bp), and the poly A tail (unknown length).

To maximize transgene expression and ensure stable field performance a large number (25 to 30) primary transformants were selected for expression levels, screened for copy number, and single-copy high-expressing lines evaluated for field performance. Standard methods (e.g. Northern and Western blotting Sambrook et al. 1989) were used to evaluate expression levels. As outlined above 30 transformants containing the native ORF5 gene and 23 transformants containing the synthetic ORF5 gene were screened by PCR, and analyzed by RT-PCR (native ORF5 gene only) and Northern blot analysis.

Northern analysis of tobacco plants transformed with pPSP-ORF5A was also carried out. Protein expression in 8 of these transgenic tobacco plants was examined by Western analysis using a polyclonal antiserum specific for the ORF5 expression product. This antibody was obtained from pigs. Of these plants comprising the PSP-ORF5A construct, 7 were found to express the ORF5 gene product (Figure 6).

TABLE 1: Summary of analysis of transgenic plants containing native ORF5

Transgenic plant	PCR Screening	Reverse transcription PCR	Northern Analysis
A	positive	positive	positive
B	positive	positive	positive
C	positive	positive	positive
D	positive	positive	positive
E	negative	negative	negative
F	positive	negative	negative
G	positive	positive	positive
H	positive	positive	positive
J	positive	positive	positive
K	not determined	negative	negative
L	positive	positive	positive
M	positive	positive	positive
N	positive	positive	not determined
P	positive	positive	positive
Q	positive	positive	negative
R	positive	positive	positive
S	positive	positive	positive
T	positive	positive	positive
V	positive	positive	positive
W	positive	positive	negative
X	positive	positive	positive
Y	positive	positive	positive
Z	positive	positive	positive
AA	positive	positive	positive
BB	positive	positive	positive
CC	positive	positive	positive
DD	positive	positive	negative
EE	positive	positive	negative
FF	positive	positive	positive
GG	not determined	positive	positive
81V9	negative	negative	negative

ELISA was used to quantify transgene protein yields. A monospecific polyclonal antibody for the ORF5 protein has been raised in rabbits using a GST-ORF5 fusion protein synthesized in E. coli. Furthermore, a polyclonal antibody raised in pigs against the whole PRRS virus has also been prepared. Both of these antibodies were used for protein expression studies as well as for ELISA for protein quantitation.

ORF5 Protein Purification

Five grams of leaf tissue from transgenic tobacco plants are quick frozen in liquid nitrogen and 1X binding buffer, filtered through Miracloth, centrifuged at 500 x g for 5 minutes and filtered again through a 0.45 micron membrane. The filtrate is passed through a chromatography column containing His-Bind Ni2+ resin, and washed with 10 vol. binding buffer, then 6 vol. wash buffer. The ORF5 protein wash is then eluted from the column with 6 vol. elution buffer, quick frozen in liquid nitrogen and stored at -70 C for later use.

Immunization Experiments of Animals:

Two routes of administration may be considered to elicit protective immunity in animals by the recombinant ORF5 protein produced in plants: intramuscular administration and oral administration.

Intramuscular administration

Four groups of pigs, three animals each group, are immunized with the ORF protein extract. The four groups are comprised of two placebo groups immunized with normal plant extracts of either alfalfa or tobacco, a group immunized with alfalfa-ORF5 protein, and a group immunized with tobacco-ORF5 protein. Animals are bled prior to first immunization, and the preimmune antibody titres for PRRS virus are determined. Antigens are mixed with oil-based adjuvants, and approximately 100 μg of ORF5 protein is used per animal for intramuscular immunization at three-week intervals for a total of

three times. Peripheral blood samples are taken at 3 weeks, 6 weeks, and 8 weeks post-immunization, and examined for the development of specific antibody responses to the ORF5 protein and PRRS virus.

Oral Administration

Strategies for oral immunization of animals against PRRS virus with the plant-produced ORF5 protein is based on the concept of common mucosal immune system. It is well documented that immunity induced on the mucosal sites of the body, especially of the intestinal tracts, is able to migrate to distal mucosal sites of the body through the common mucosal immune system (reviewed in Kagnoff, 1996). In humans, adenovirus infects respiratory tracts and causes acute respiratory illness. This disease is especially referred to acute respiratory disease (ARD) in the military personnel. Adenovirus vaccine has been developed and used as an oral vaccine to prevent ARD in the US Army over the past 40 years. This approach has been proven very effective to induce mucosal immunity in the respiratory tract by oral administration of the vaccine antigen. Since primary infection site in pigs by PRRS virus is the respiratory tract, it may be very important to induce neutralizing antibody responses in the respiratory tract to prevent the infection effectively. By administering the ORF5 antigen onto the intestinal sites in pigs, effective immunity may be induced on the respiratory sites, thereby preventing PRRS virus infections. Four groups of pigs, three animals each group, are used for feeding experiments. Two groups of animals are orally fed with normal plants (tobacco and alfalfa), and two other groups of animals are fed with transgenic plants expressing the ORF5 protein. Blood samples are collected from the animals at 3 weeks and 6 weeks post-immunization to monitor the antibody responses in the circulation system. Antibody responses may also be examined in the guts and respiratory tract. Upon appearance of antibody titres in the sera, the animals are sacrificed and gut washes and lung lavages are collected for secretory antibody analysis.

Analysis of specific antibodies

Serum samples are analysed by enzyme-linked immuno sorbent assay (ELISA) for the presence of specific antibodies to ORF5 protein. PRRS virus is grown in tissue culture and purified through discontinuous sucrose gradient centrifugation. The viral antigens are coated on the ELISA plates, and the IgG detection ELISA is performed. The serum samples are also examined for their ability to neutralize virus infectivity by plaque reduction neutralization assays. Lung lavages and gut washes are examined for the presence of secretory IgA antibodies and neutralizing titers. For detection of IgA antibodies, PRRS virus-coated plates are incubated with the gut washes or lung lavages at various dilutions, and the antigen-antibody complexes are incubated with the secondary anti-porcine IgA antibody. Anti-secondary antibody conjugate and chromogenic substance are added for colour developments. Ability of the secretory IgA for virus neutralization is also examined by PRRS virus plaque reduction assays.

All references cited herein are incorporated by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described in the following claims

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of preparing at least one porcine reproductive and respiratory syndrome virus protein comprising:

   i) obtaining a gene encoding the protein;
   ii) inserting the gene within a vector suitable for transforming a plant;
   iii) transforming a plant with the vector; and
   iv) growing the transformed plant.

2. The method of claim 1 wherein the gene is a native gene sequence.

3. The method of claim 1 wherein the gene is a synthetic gene sequence.

4. The method of claim 2 wherein the native gene sequence is modified in order to enhance expression of the gene and stability of the protein within plant tissues.

5. The method of claim 4 wherein the modification includes replacing the native signal sequence with a heterologous signal sequence.

6. The method of claim 5 wherein the heterologous signal sequence is the PR1b sequence.

7. The method of claim 4 wherein the modification includes an ER retention motif.

8. The method of claim 4 wherein the modification includes a cleavage site.

9. The method of claim 7 wherein the modification further includes a HIS tag.

10. The method of claim 3 wherein the synthetic gene sequence is modified in order to enhance expression and stability of the gene within plant tissues.

11. The method of claim 10 wherein the native gene sequence is modified in order to enhance expression of the gene and stability of the protein within plant tissues.

12. The method of claim 10 wherein the modification includes replacing the native signal sequence with a heterologous signal sequence.

13. The method of claim 12 wherein the heterologous signal sequence is the PR1b sequence.

14. The method of claim 10, wherein the modification includes an ER retention motif.

15. The method of claim 10, wherein the modification includes a cleavage site.

16. The method of claim 15 wherein the modification further includes a HIS tag.

17. The method of claim 1 wherein the gene encodes the ORF 5 protein.

18. The method of claim 1 wherein the plant is a tobacco plant.

19. The method of claim 1 wherein the plant is an alfalfa plant.

20. The method of claim 1 wherein the protein is extracted from harvested plant tissues and purified.

21. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:1.

22: A vector comprising the DNA molecule of claim 21 operatively associated with a promoter, enhancer and terminator regions.

23. A plant comprising the vector of claim 22.

24. A protein produced by the method of claim 1.

25. A protein produced by the method of claim 20.

26. A method of immunizing pigs against porcine reproductive respiratory syndrome comprising:

i) transforming a plant with the vector of claim 22;
ii) growing the transformed plant;
iii) feeding tissues of the transformed plant to pigs
iv) repeating step iii) as needed.

27. The method of claim 26, wherein the step of feeding the tissues of the transformed plant to pigs comprises grazing un-harvested tissues.

28. The method of claim 26, wherein prior to step iii), tissues of the plant are harvested, and the harvested tissues are feed to the pig.

29. A method of immunizing pigs against porcine reproductive respiratory syndrome comprising:

i) transforming a plant with the vector of claim 22;
ii) growing the transformed plant;
iii) harvesting the transformed plant to obtain harvested tissue;
iv) extracting the protein encoded by the DNA molecule from the harvested tissue;

v) administering the protein to the pig;

vi) repeating step v) as needed.

30. The method of claim 29 wherein in step v), the protein is administered to the pig as a food supplement, or as an injection.

31. The method of claim 1, wherein tissues are harvested from the transformed plant to obtain harvested tissue.

32. The method of claim 31, wherein the protein is extracted from the harvested tissue.

33. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:2.

34. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:3.

35. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:4.

36. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:5.

37. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:6.

38. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:7.

39. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:8.

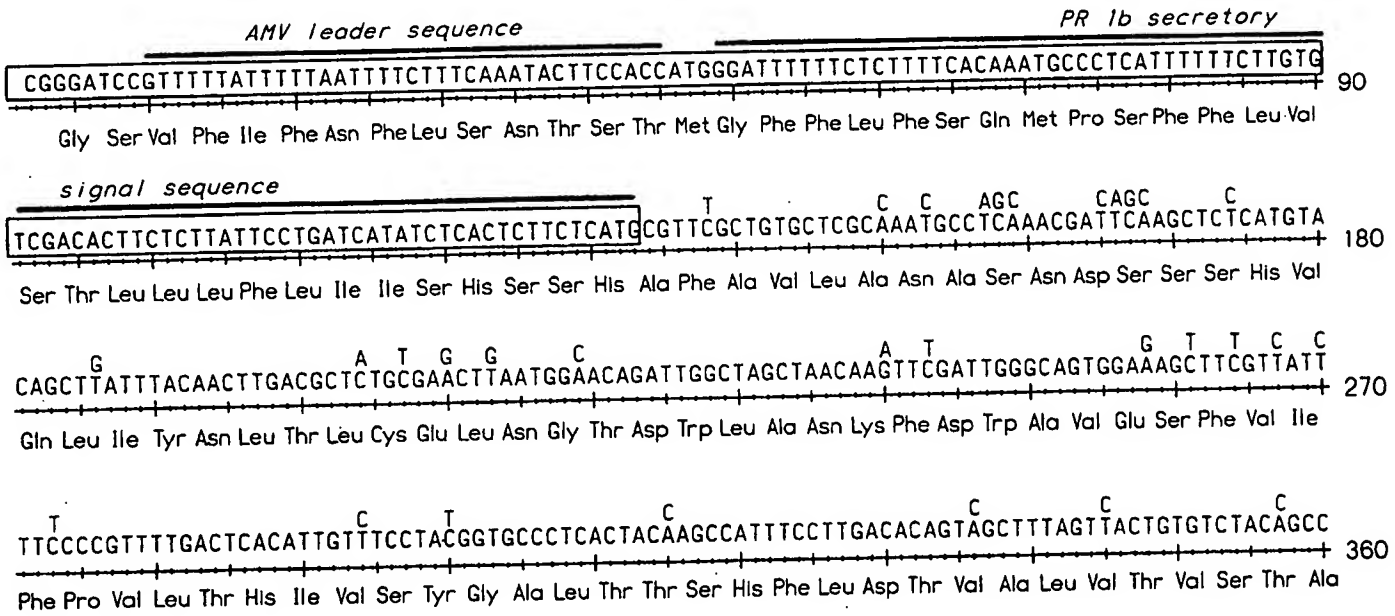
40. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:9.

41. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:10.

42. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:11.

43. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:12.

44. An isolated DNA molecule comprising the sequence defined by SEQ ID NO:13.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Gly Ser Val Phe Ile Phe Asn Phe Leu Ser Asn Thr Ser Thr Met Gly Phe Phe Leu Phe Ser Gln Met Pro Ser Phe Phe Leu Val
signal sequence
TCGACACTTCTCTTATTCCTGATCATATCTCACTCTTCTCATGCGTTCGCTGTGCTCGCAAATGCCTCAAACGATTCAAGCTCTCATGTA 180
Ser Thr Leu Leu Leu Phe Leu Ile Ile Ser His Ser Ser His Ala Phe Ala Val Leu Ala Asn Ala Ser Asn Asp Ser Ser Ser His Val
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Gln Leu Ile Tyr Asn Leu Thr Leu Cys Glu Leu Asn Gly Thr Asp Trp Leu Ala Asn Lys Phe Asp Trp Ala Val Glu Ser Phe Val Ile
TTCCCCGTTTTGACTCACATTGTTTCCTACGGTGCCCTCACTACAAGCCATTTCCTTGACACAGTAGCTTTAGTTACTGTGTCTACAGCC 360
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FIGURE 1

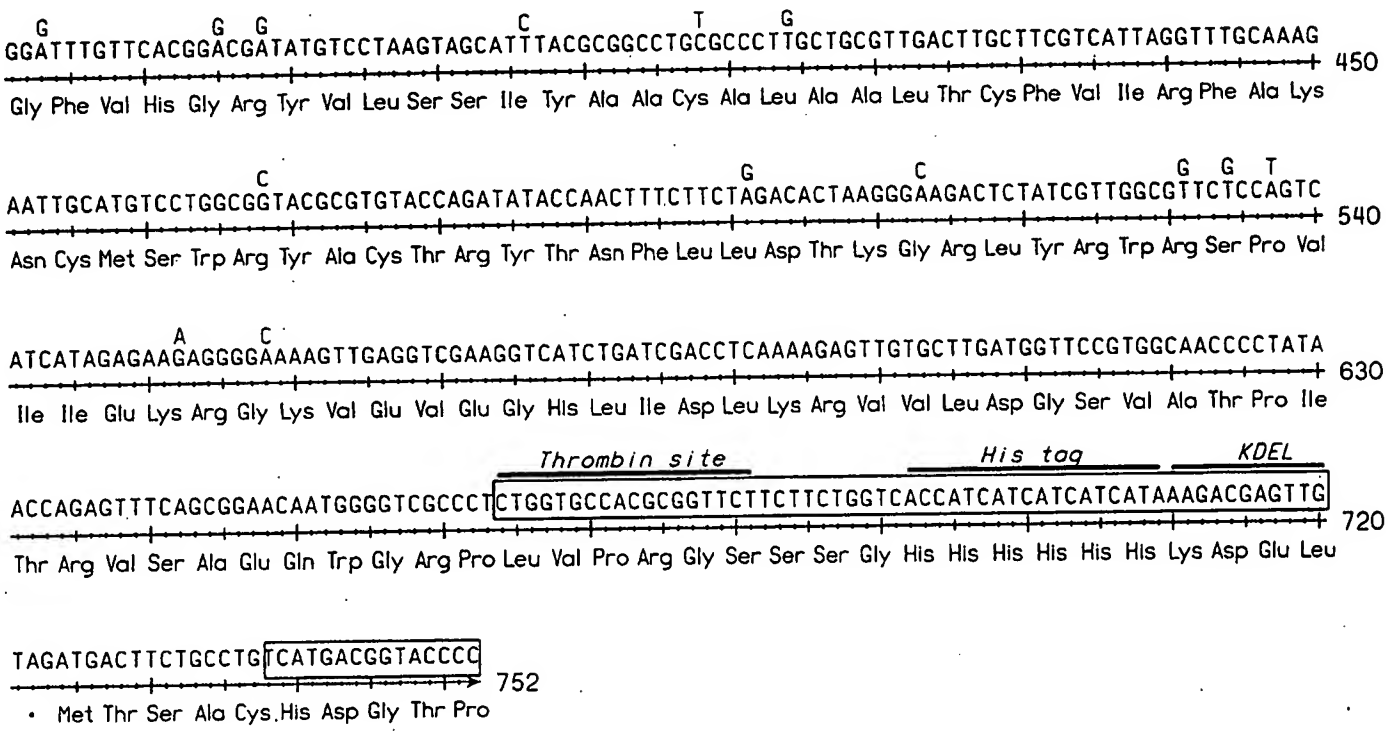

FIGURE 1 (Cont'd)

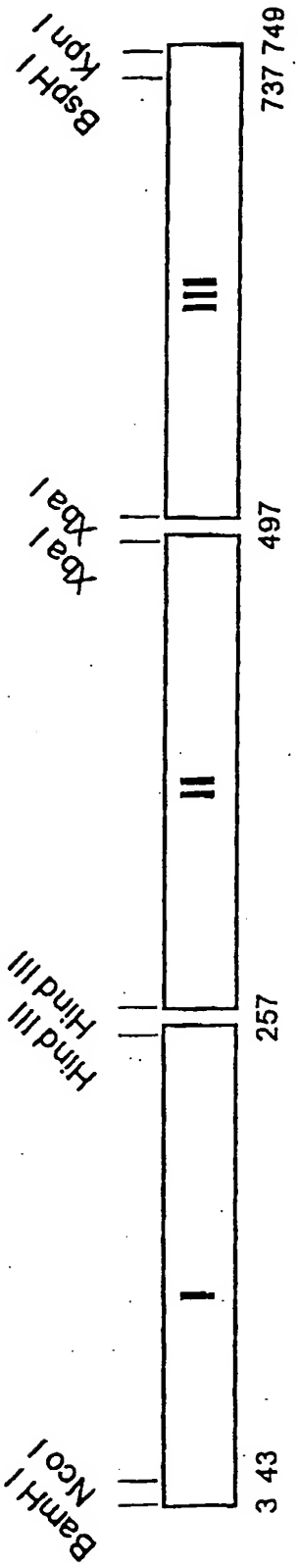
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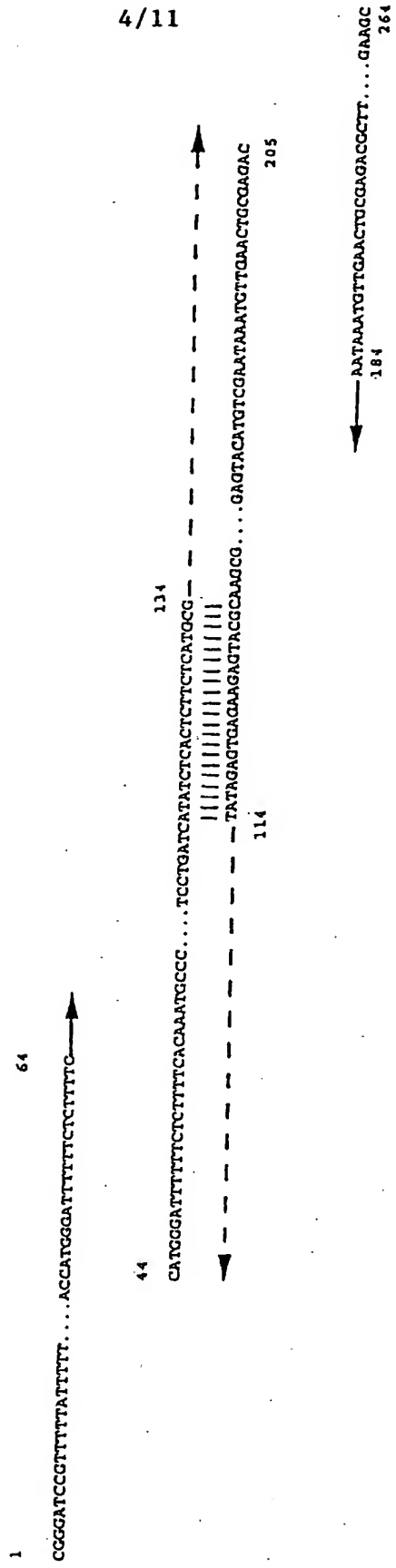
FIG. 2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TATAGAGTGAGAAGAGTACGGCAAGCG....GAGTACATGTCGAATAAATGTTGAACTGGCGAGAC
114
205
AATAAATGTTGAACTGCGAGACGCTT....GAAGC
184
264

FIG. 3

Block I

Primer1:

5'CGGGATCCGTTTTTATTTTTAATTTTCTTTCAAATACTTCCACCATGGGATTTTTTCT
CTTTTC

Primer 2:

5'CATGGGATTTTTTCTCTTTTCACAAATGCCCTCATTTTTTCTTGTGTCGACACTTCTC
TTATTCCTGATCATATCTCACTCTTCTCATGCG

Primer 3:

5'CAGAGCGTCAAGTTGTAAATAAGCTGTACATGAGAGCTTGAATCGTTTGAGGCATT
TGCGAGCACAGCGAACGCATGAGAAGAGTGAGATAT

Primer 4:

5'CGAAGCTTTCCACTGCCCAATCGAACTTGTTAGCTAGCCAATCTGTTCCATTAAGTT
CGCAGAGCGTCAAGTTGTAAATAA

FIGURE 4

Block II

Primer 5:

5'GGAAAGCTTCGTTATTTTCCCCGTTTTGACTCACATTGTTTCCTACGGTGCCCTCAC

TACAAGCCATTTCC

Primer 6:

5'CCCTCACTACAAGCCATTTCCTTGACACAGTAGCTTTAGTTACTGTGTCTACAGCCG

GATTTGTTCACGGACGATATGTCCTAAGTAGC

Primer 7:

5'GCCAGGACATGCAATTCTTTGCAAACCTAATGACGAAGCAAGTCAACGCAGCAAG

GGCGCAGGCCGCGTAAATGCTACTTAGGACATATCGTCCG

Primer 8:

5'GTGTCTAGAAGAAAGTTGGTATATCTGGTACACGCGTACCGCCAGGACATGCAATT

CTTTG

FIGURE 4 (Cont'd)

Block III

Primer 9:

5'ACCAACTTTCTTCTAGACACTAAGGGAAGACTCTATCGTTGGCGTTCTCCAGTCAT

CATAGAGAAGAGGGGAAAAGTTGAGG

Primer 10:

5'AGAGAAGAGGGGAAAAGTTGAGGTCGAAGGTCATCTGATCGACCTCAAAAGAGTT

GTGCTTGATGGTTCCGTGGCAACC

CCTATAACCAG

Primer 11:

5'ATGATGATGATGGTGACCAGAAGAAGAACCGCGTGGCACCAGAGGGCGACCCCAT

TGTTCCGCTGAAACTCTGGTTATAGGGGTTGCCACG

Primer 12:

5'CCGGGGTACCGTCATGACAGGCAGAAGTCATCTACAACTCGTCTTTATGATGATGA

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FIGURE 4 (Cont'd)

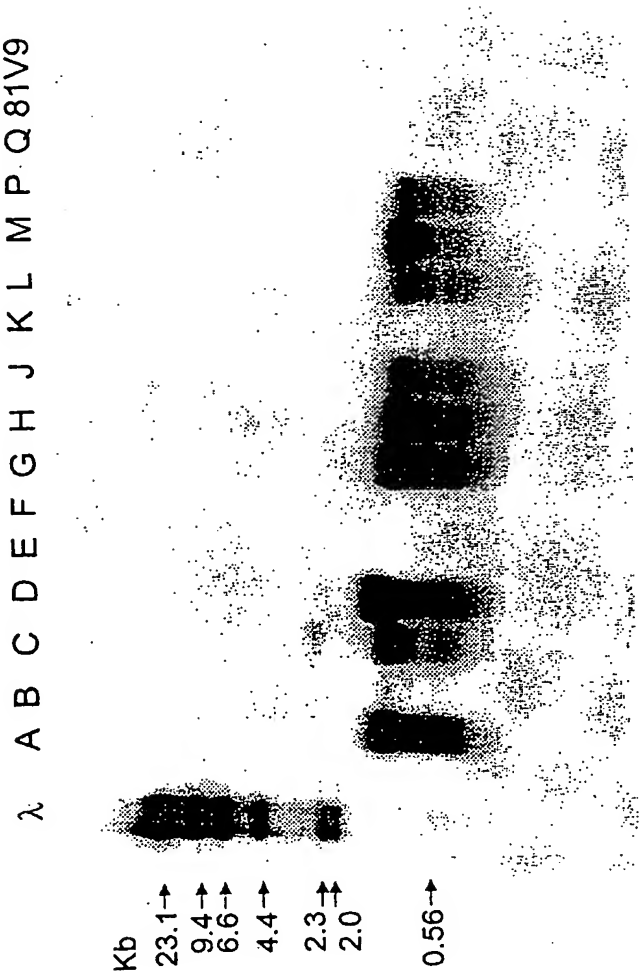


FIGURE 5A

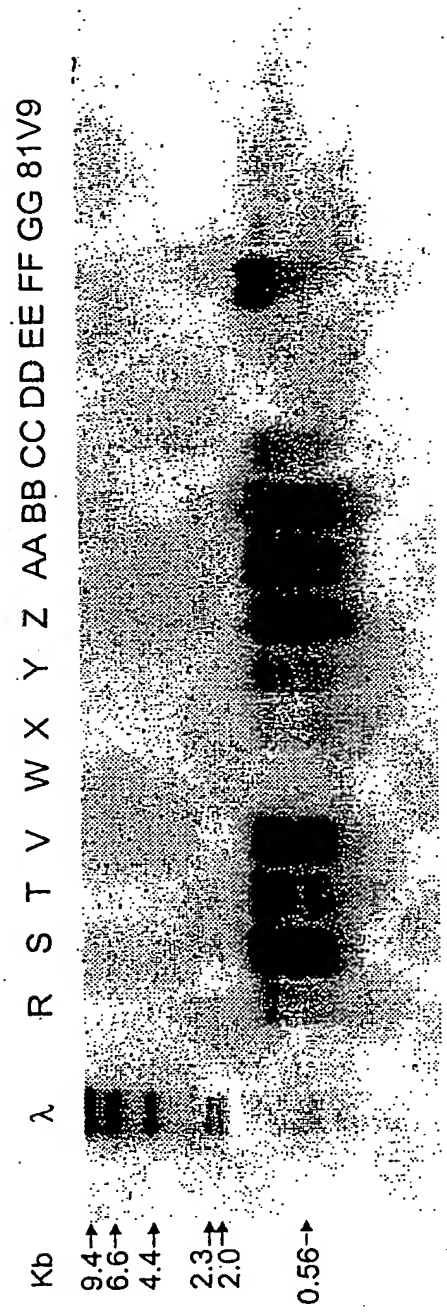


FIGURE 5B

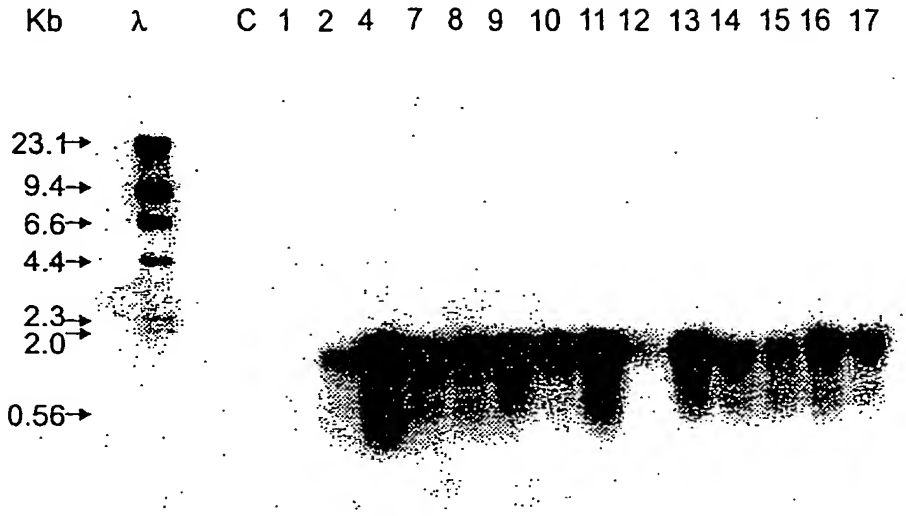


FIGURE 5C

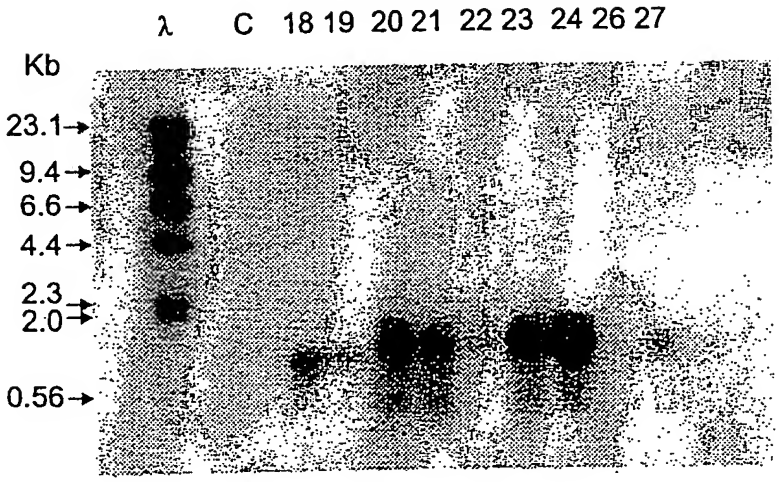
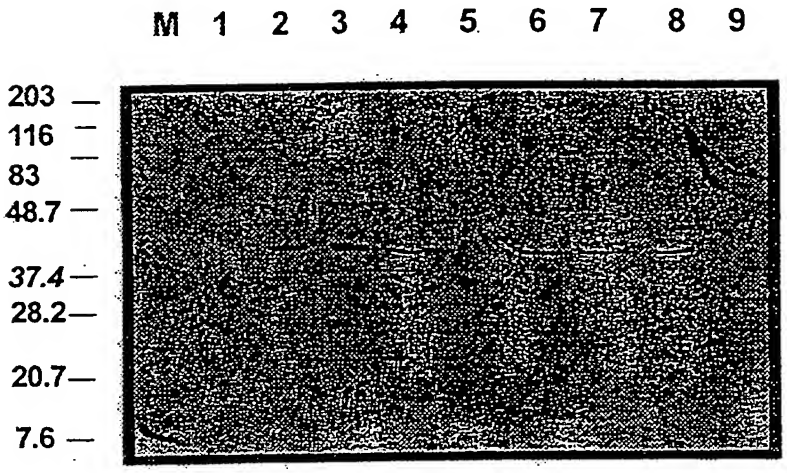


FIGURE 5D

Western blot probed with the polyclonal antiserum specific for ORF-5



M-Prestained SDS-PAGE standards (Broad range)
1-Control plant,
2-9 transgenic plants

FIGURE 6

SEQUENCE LISTING

<110> Her Majesty in Right of Canada as Represented by Agriculture and Agri-Food Canada and the University of Guelph

<120> Porcine Reproductive and Respiratory Syndrom Oral Vaccine Production in Plants

<130> PRRS- Ag Can/U of Guelph

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<150> 2,221,843
<151> 1998-01-27

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ggtgaccaga ag 72

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/CA 99/00064

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/40 C12N15/82 C07K14/08 A61K39/12 A01H5/00
C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANA DURAN, J. ET AL.: "Baculovirus expression of proteins of Reproductive and Respiratory Syndrome Virus strain Olot/91. Involvement of ORF3 and ORF5 proteins in protection" VIRUS GENES, vol. 14, no. 1, 1997, pages 19-29, XP002103647	24,25
Y	see page 20 - page 27 'Materials and Methods' and 'Results'	26-30

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PIRZADEH, B. AND DEA, S.: "Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants" JOURNAL OF GENERAL VIROLOGY, vol. 78, August 1997, pages 1867-1873, XP002103648	24,25
Y	see the whole document	1-20,31, 32

X	ES 2 096 529 A (UNIVERSIDAD COMPLUTENSE DE MADRID) 1 March 1997	24,25
Y	see column 2, line 50 - column 4, line 59	1-4, 9-11, 16-18

Y	MASON, H.S. ET AL.: "Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice" PROC.NATL.ACAD.SCI.USA, vol. 93, May 1996, pages 5335-5340, XP002025358 cited in the application see the whole document	1-20, 26-32

Y	HAQ, T.A. ET AL.: "Oral immunization with recombinant bacterial antigen produced in transgenic plants" SCIENCE, vol. 268, May 1995, pages 714-716, XP002024034 cited in the application see the whole document	1-20, 26-32

Y	DENECKE J. ET AL.: "Protein secretion in plant cells can occur via a default pathway" THE PLANT CELL, vol. 2, January 1990, pages 51-59, XP002093514 cited in the application see page 52 - page 56 'Results'	1-6,8, 10-13, 15,18

	-/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHOUTEN, A. ET AL.: "The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco" PLANT MOLECULAR BIOLOGY, vol. 30, 1996, pages 781-793, XP000677225 cited in the application see page 786 - page 791 'Results' and 'Discussion'	1-5,7,8, 10-12, 14,15,18
A	MIELE, L.: "Plants as bioreactors for biopharmaceuticals: regulatory considerations" TIBTECH, vol. 15, February 1997, pages 45-50, XP004034112 cited in the application see the whole document	1-20, 26-32
A	CONZELMANN, K.-K. ET AL.: "Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the Arterivirus group" VIROLOGY, vol. 193, 1993, pages 329-339, XP002084424 see the whole document	1-25, 33-44

INTERNATIONAL SEARCH REPORT

information on patent family members

Int. Application No
PCT/CA 99/00064

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
ES 2096529 A	01-03-1997	NONE	